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# Toward Fluorescence-Based High-Throughput Screening for Enantiomeric Excess in Amines and Amino Acid Derivatives

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**Abstract:** A highly accurate and reliable screening method for enantiomeric excess of amine derivatives in the presence of water is reported. The fluorescence-based screening system has been realized by self-assembly of chiral diol-type dyes (BINOL, VANOL and VAPOL), 2-formylphenylboronic acid, and chiral amines forming iminoboronate esters. Structure and chirality of the amine analytes determine the stability of the diastereomeric iminoboronate esters, which in turn display differential fluorescence. The fluorescence signal reflects the enantiomeric purity of the chiral amines and was utilized in high-throughput arrays. The arrays were able to recognize enantiomeric excess of amines, amino esters, and amino alcohols. In addition to qualitative analysis, quantitative experiments were successfully performed. Studies of the role of additives such as water or citrate were carried out to gain insight into the stability of the iminoboronate esters. It is shown that the above additives destabilize less stable esters while the stable esters remain unchanged. Thus, the presence of water and citrate leads to increased difference between the diastereomeric iminoboronates and contributes to the enantiodiscrimination of the chiral amines.

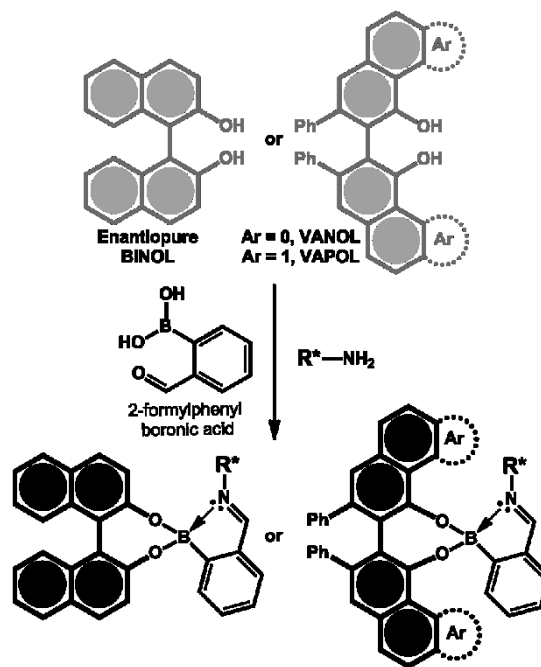
## Introduction

Chiral amines are important biologically active compounds,<sup>1</sup> chiral auxiliaries,<sup>2</sup> as well as valued synthons in asymmetric synthesis either as starting materials or ligands and catalysts for a number of asymmetric transformations.<sup>3</sup> Furthermore, chiral amines are used in pharmaceutical chemistry as drugs and drug precursors.<sup>4</sup> Chiral amines are available from the chiral pool or from enantioselective syntheses.<sup>5</sup> Due to their importance, analysis of enantiomeric purity and determination of enantiomeric excess (e.e.) play a pivotal role in organic and pharmaceutical chemistry, and the development of methods for rapid and accurate determination of e.e. are highly desirable.<sup>6</sup> Typical analyses of the enantiomeric purity require GC and HPLC with chiral columns,<sup>7</sup> the measurement of optical rotation,<sup>8</sup> or NMR spectroscopy using chiral shift reagents.<sup>9</sup> Recently, optical

methods such as UV-Vis spectroscopy using chiral probes<sup>10</sup> and circular dichroism (CD)<sup>11</sup> spectroscopy were brought to high-throughput level.<sup>12</sup> However, with few exceptions,<sup>13</sup> these methods are not suitable for high throughput-screening (HTS) and, despite significant effort, the development of methods for the determination of enantiomeric purity in a high-throughput fashion are in their early stages.

Molecular self-assembled systems utilizing boronate esters have been widely employed in fields as disparate as saccharide recognition,<sup>14</sup> catalysis,<sup>15</sup> molecular sensing,<sup>16</sup> drug delivery,<sup>17</sup> development of stimuli responsive materials,<sup>18</sup> light emitters,<sup>19</sup> organic electronic materials,<sup>20</sup> and self-healing materials.<sup>21</sup> Iminoboronates are of particular interest as excellent molecular building blocks<sup>22</sup> for covalent dynamic assemblies<sup>23</sup> which found application in sensing of enantiomeric excess<sup>24</sup> in amines<sup>25</sup> and diols.<sup>26</sup>

Recently, James and Bull reported that a ternary system consisting of enantiomerically pure 1,1'-bi-2-naphthol (BINOL), 2-FPBA, and an  $\alpha$ -chiral primary amine assembles upon mixing to form diastereomeric iminoboronates that display characteristic <sup>1</sup>H NMR spectroscopic shifts corresponding to the amine enantiomer used to establish the iminoboronate.<sup>27</sup> More recently, we have demonstrated that iminoboronates comprising



**Scheme 1.** Self-assembly formation between a diol-type fluorophore, 2-FPBA, and a chiral amine or amine derivative.

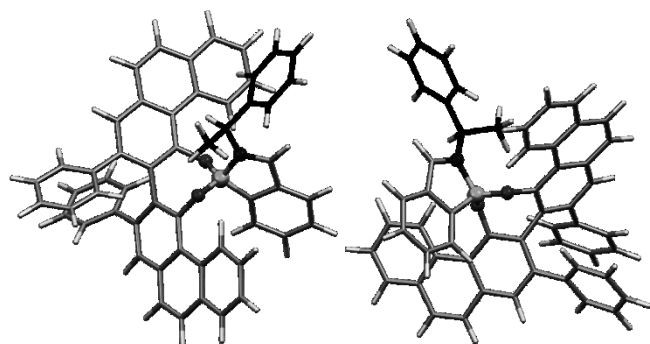
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2-FPBA, chiral amines and enantiopure fluorescent diols such as BINOL, VANOL or VAPOL (Scheme 1) show differential fluorescence for the respective amine enantiomers.<sup>28</sup> We believe that this differential fluorescence signal is useful in high-throughput determination of enantiomeric excess (e.e) using a simple microplate fluorescence reader. While successful in differentiation of amines carrying aromatic residues, the performance in differentiation of aliphatic amines and aliphatic amino esters was found lacking. The present study reports on our current efforts to remedy this deficiency.

## Results and Discussion

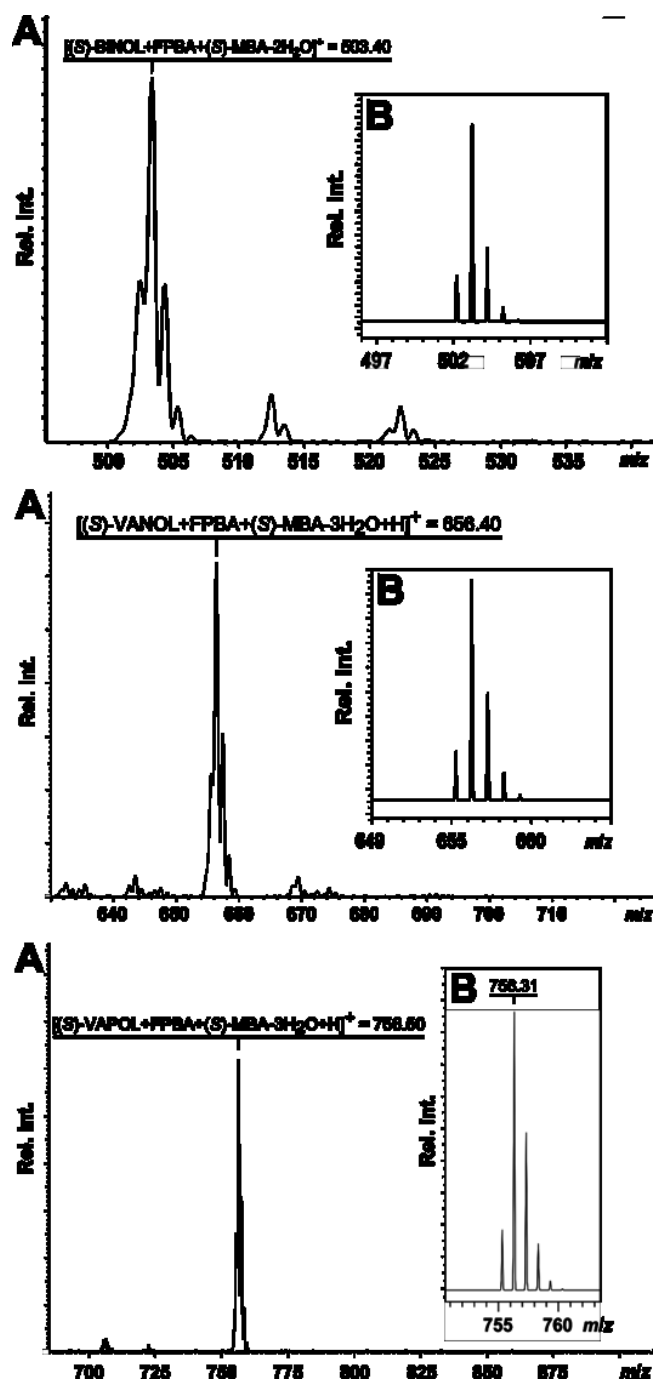
With this research we develop a deeper insight into the amine-2-FPBA-diol systems and their utility for the analysis of enantiomeric excess in amines. With our preliminary report,<sup>28</sup> we have shown the X-ray crystal structures of the enantiomeric pair of iminoboronate esters (*S,S* and *R,R*) self-assembled from FPBA,  $\alpha$ -methylbenzylamine (MBA) and VAPOL (1:1:1 stoichiometry), which display the pseudotetrahedral geometry of the boron atom coordinated to the imine nitrogen.



**Figure 1.** The DFT optimized structures of two diastereomers: (left) (*S,S*)- and (right) (*R,R*)-iminoboronate esters self-assembled from FPBA, MBA, and VAPOL. Calculations were performed in Gaussian '09.<sup>29</sup>

Here, we have performed density functional theory (DFT) calculations of diastereomeric and enantiomeric couples that reveal the formation of complexes in 1:1:1 stoichiometry of a fluorescent chiral diol (VANOL or VAPOL), 2-FPBA, and a chiral amine. The molecules were optimized at the DFT level of theory with the B3LYP functional and the 6-31G\* basis set. The effect of the acetonitrile solvent was included implicitly using the Polarizable Continuum Model (PCM). DFT-optimized geometries match well the corresponding single-crystal X-ray structures.

The calculated geometries show the same dihedral angle which defines the “twisting” of the two phenanthrene (VAPOL) or naphthalene (VANOL) planes of the fluorescent ligands among couples of enantiomers and diastereomers as well (53.86°, 53.78° for VANOL, and 60.17°, 59.98° for VAPOL). However, the dihedral angles corresponding to tetrahedral arrangement around the boron center (O1–B–N–C\* and O2–B–N–C\*) in two



**Figure 2.** Top: (A) ESI MS of the iminoboronate ester self-assembled from FPBA, (*S*)-MBA, and (*S*)-BINOL. (B) Calculated isotope pattern for  $C_{35}H_{26}BNO_2^+$ . Middle: (A) ESI MS of the iminoboronate ester self-assembled from FPBA, (*S*)-MBA, and (*S*)-VANOL. (B) Calculated isotope pattern for  $C_{47}H_{35}BNO_2^+$ . Bottom: (A) ESI MS of the iminoboronate ester self-assembled from FPBA, (*S*)-MBA, and (*S*)-VAPOL. (B) Calculated isotope pattern for  $C_{55}H_{39}BNO_2^+$ . All spectra confirmed the formation of self-assemblies with 1:1:1 stoichiometry.

diastereomeric couples are different compared to the pairs of enantiomers (64.99°, 81.08° and -58.52°, -43.08° for VANOL, or

63.80°, 79.45°, and -59.93°, -45.08° for VAPOL). From the DFT models one can see that the analyte-ligand complex displays a different geometry around the boron center (see Supporting Information), which results in different stabilities of the iminoboronate. This in turn induces changes in fluorescence intensity and energy.

Electrospray ionization (ESI) mass spectrometry was employed as further evidence of the stoichiometry of the self-assembled complexes of chiral amines. ESI MS confirmed the successful formation of self-assemblies of the diol-fluorophore, FPBA and analytes with 1:1:1 stoichiometry (Fig. 2).

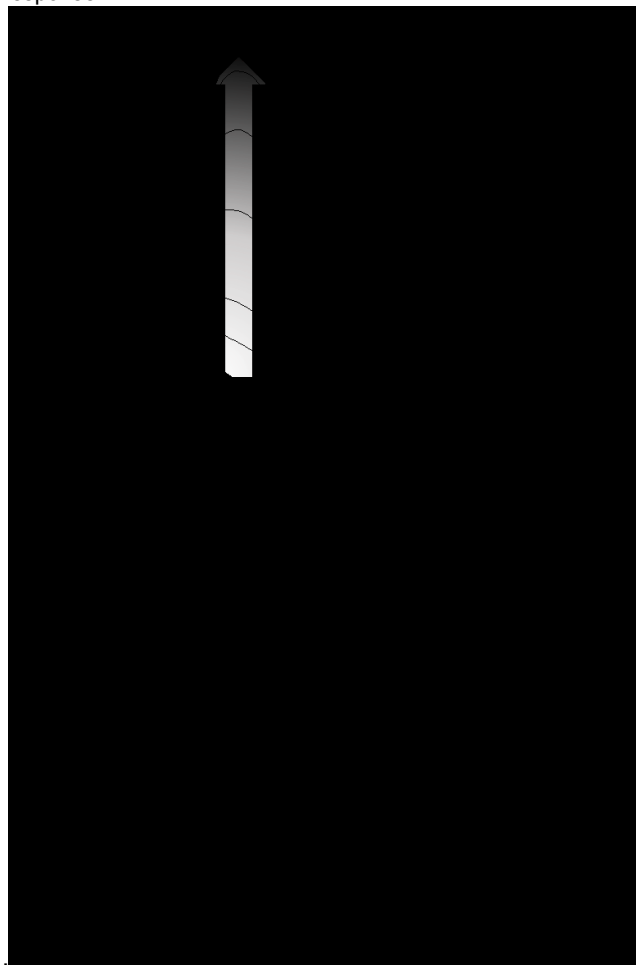
Next, fluorescence titrations with amines, amino esters, and amino alcohols were performed. As guest analytes, we selected 2-aminobutane (AMB), methylbenzylamine (MBA), methylphenethylamine (MPA), 1-(2-naphthyl)ethylamine (NEA), *cis*-1-amino-2-indanol (AID), 2-amino-3-phenyl-1-propanol (APP), and methyl esters of alanine (Ala-OMe), phenylalanine (Phe-OMe), tryptophan (Trp-OMe), valine (Val-OMe), and methionine (Met-OMe) (Figure 3).



**Figure 3.** Molecular structures of target analytes.

The titrations were performed in MeCN, and the diastereomeric iminoboronates displayed interesting differences in fluorescence signal. In the case of amine addition, fluorescence quenching was observed. Similarly, the response to amino esters exhibited fluorescence quenching. This quenching is ascribed to a photoinduced electron transfer process arising from the nitrogen.<sup>30</sup> However, amino alcohols such as aminophenyl propanol (APP) display ratiometric changes in fluorescence. Previously, James et al. reported that BINOL with FPBA can form oxazolidine boronate esters with chiral amino alcohols.<sup>31</sup> This

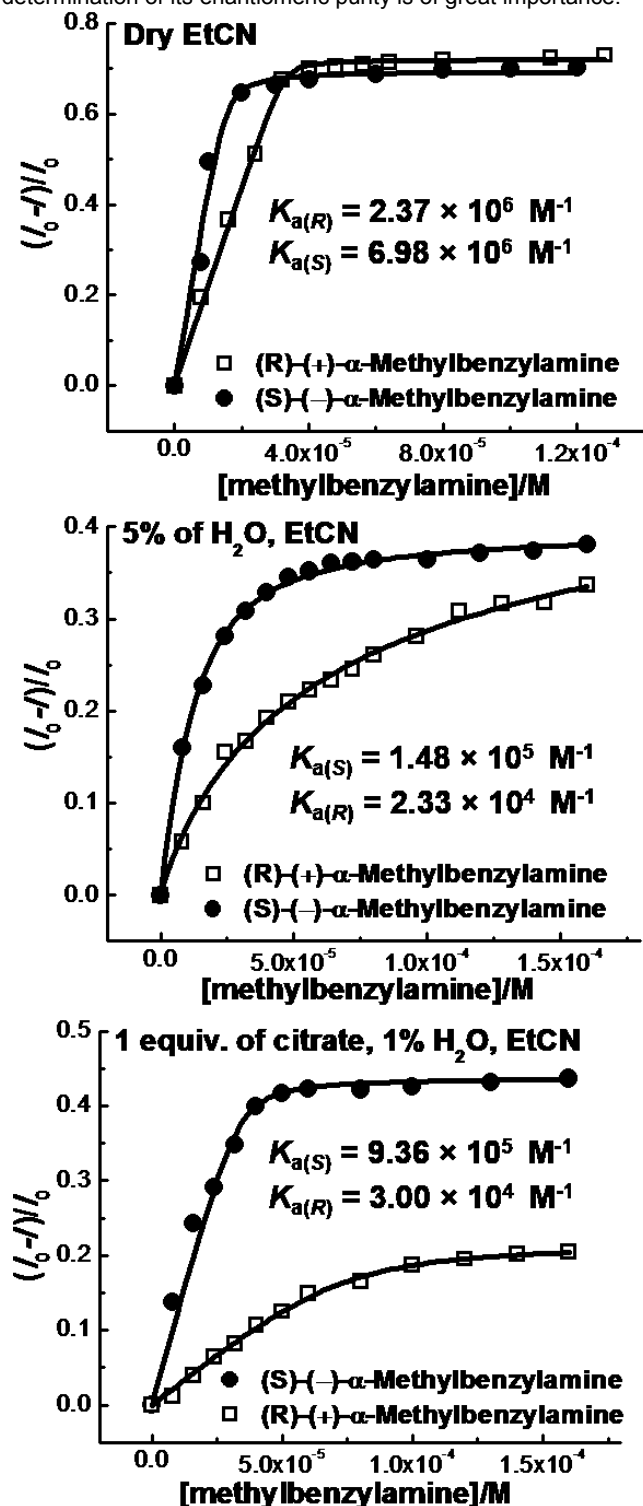
might be the reason for the difference in the observed fluorescent response.



**Figure 4. Top:** Titration profile of 1 equiv. of (S)-VANOL with 1 equiv. of 2-formylphenylboronic acid (40  $\mu$ M) upon the addition of incremental amounts of (1S,2R)-(-)-*cis*-1-amino-2-indanol in acetonitrile.  $\lambda_{\text{ex}}$  = 335 nm. [(1S,2R)-(-)-*cis*-1-amino-2-indanol] = 0 - 100  $\mu$ M. **Bottom:** Comparison of two isotherms for the two enantiomers of *cis*-1-amino-2-indanol.

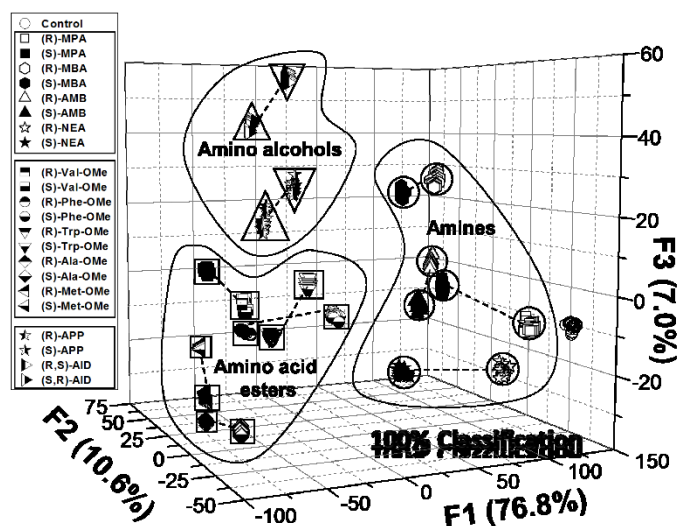
In general, one of the iminoboronate diastereomer showed a larger change in fluorescence response than the other, depending on the structure of the chiral amine. In addition, the value of the apparent binding constant is in most cases different for each amine enantiomer. In other words, the configuration of the chiral amine influences the stability of the resulting iminoboronate complex and this stability is reflected in the fluorescence output. Fig. 4 top shows the fluorescence spectra of the iminoboronate ester self-assembled from VANOL, FPBA, and different concentrations of (1S,2R)-(-)-*cis*-1-amino-2-indanol, as well as the corresponding binding isotherm. Figure 4 bottom shows the isotherms for both enantiomers, displaying a strong fluorescence signal amplification and a large difference between (1S,2R)-(-)-*cis*-1-amino-2-indanol and (1R,2S)-(-)-*cis*-1-amino-2-indanol. Enantiomerically pure (1S,2R)-(-)-*cis*-1-amino-2-indanol is a key intermediate for the synthesis of Indinavir, a widely used protease

inhibitor of human immunodeficiency virus (HIV),<sup>32</sup> and thus the determination of its enantiomeric purity is of great importance.



**Figure 5.** The binding isotherms for (S)-BINOL (40  $\mu\text{M}$ ) with FPBA (40  $\mu\text{M}$ ) upon the addition of (S)- and (R)-methylbenzylamine in different conditions. **Top:** Dry EtCN. **Middle:** 5% of water ( $\text{H}_2\text{O}$ ) in EtCN. **Bottom:** 1 equiv. of citrate used as an additive in EtCN with 1% of  $\text{H}_2\text{O}$ .

In contradistinction, some amines such as methylbenzylamine and particularly aliphatic amines such as 2-aminobutane and aliphatic amino esters (Ala-OMe, Val-OMe, Met-OMe) could not be successfully resolved by the diol-2-FPBA-amine system. In order to achieve resolution of the enantiomers of these amines, we performed a series of experiments aimed at destabilizing the formed diastereomeric iminoboronates. The formation of the iminoboronates requires dynamic covalent bond formation.<sup>23</sup> Therefore, addition of water, one of the products from the boronate esterification, results in a change in the esterification equilibrium, and this is reflected by smaller values for the apparent association constants. Additives also always destabilize the formation of one of the two diastereomeric iminoboronates more than the other, effectively creating a large difference in recognition of the amine enantiomers. For example, the diol-2-FPBA-methylbenzyl amine (MBA) in dry propionitrile (Fig. 5 top) does not show an appreciable difference between *R*- and *S*-MBA. However, the presence of water (Fig. 5, center) destabilizes the formation of the iminoboronate formed from the *R*-methylbenzylamine (MBA) over *S*-MBA. Likewise, the presence of water and citric acid as a competitor and a catalyst for the dynamic formation of the iminoboronate and its hydrolysis preferentially destabilizes the iminoboronate formed from *R*-MBA (Fig. 5, bottom). As expected, the apparent association constants ( $K_{\text{assoc}}$ ) values are different between amine enantiomers. These differences are expected to produce discrimination among different analytes in an array-based assay. Other competitors for iminoboronate formation were also tested with varying success. Among the additives tested were water, ethylene glycol, glucose, glycerol, citric acids and their mixtures.



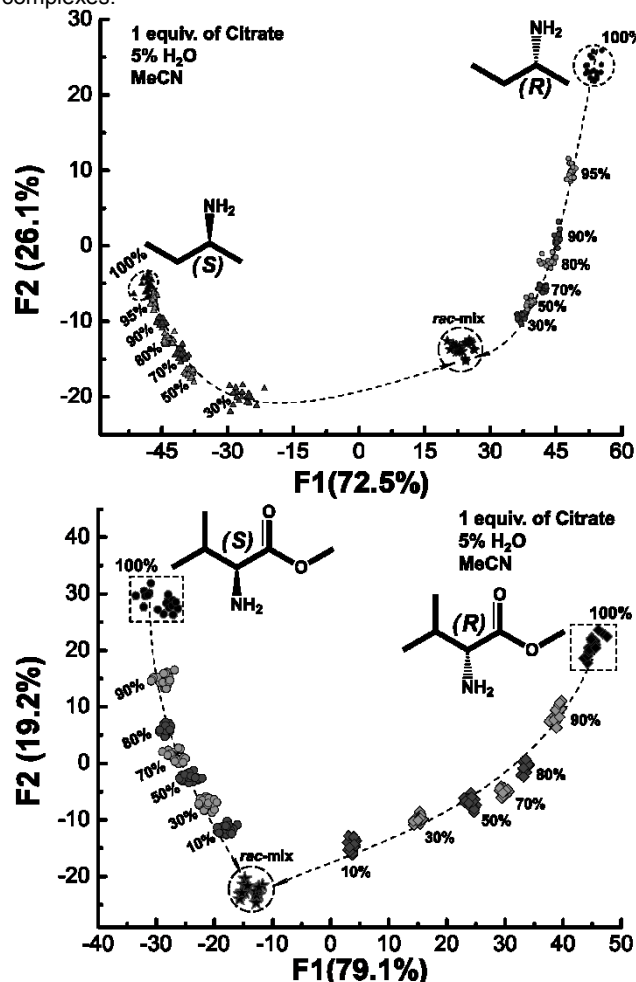
**Figure 6.** The qualitative linear discriminant analysis (LDA) of amine, amino alcohol, and amino acid ester enantiomers in  $\text{H}_2\text{O} : \text{MeCN} = 3 : 97$ , v/v with 1.5 equiv. of citrate (60  $\mu\text{M}$ ) as an additive using a fluorescence assay.

As expected, the actual values of the apparent association constants ( $K_{\text{assoc}}$   $\text{M}^{-1}$ , see Supporting Information) for amines, amino esters, and amino alcohols tested were lower in the

presence of water and water-citric acid compared to water or pure propionitrile. Thus, affinity was sacrificed for increased *R/S*-selectivity.

To confirm that the above method is suitable for *e.e.* determination in high-throughput screening, the fluorescence data derived from the boronate esterification were acquired using 384-well microplates and a fluorescence plate reader (see SI). Pattern recognition procedures were then adopted for the evaluation of the discriminatory power of the sensing system.

First, a qualitative analysis for 11 enantiomeric pairs (Fig. 3) was performed using the following conditions: 40  $\mu$ M and 1:1:1 stoichiometry of the analyte, the diol-dye, and FPBA. The qualitative analysis (Fig. 6) was performed utilizing the different emission between the diastereoisomeric iminoboronate complexes.



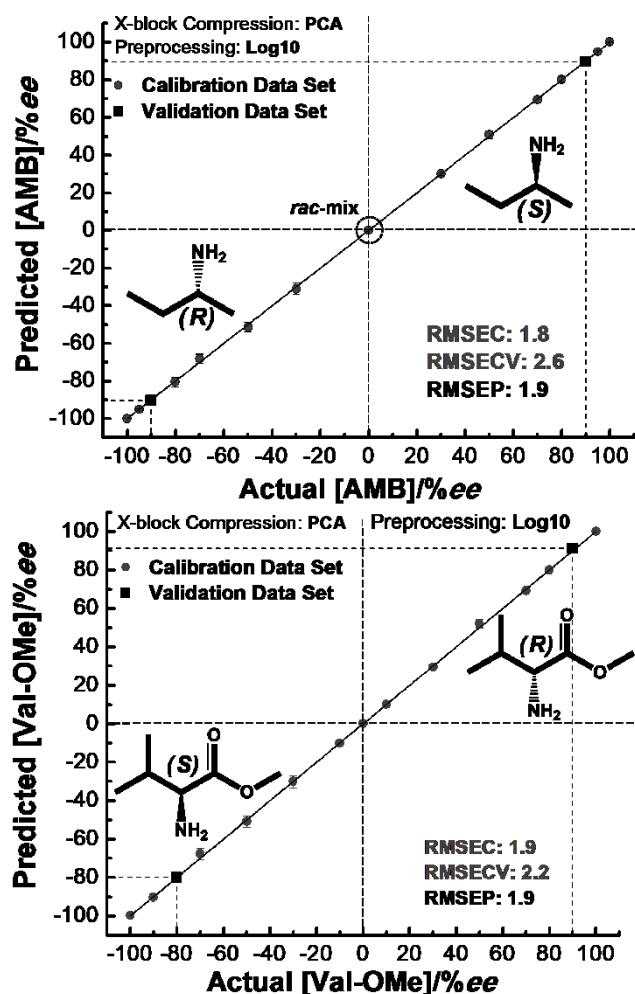
**Figure 7.** Linear discriminant analysis (LDA) describing a semiquantitative assay of enantiomeric composition of 2-aminobutane (top) and valine methyl ester (bottom) by employing the microarray. [FPBA] = 40  $\mu$ M, [Diol dye] = 40  $\mu$ M, [analyte] = 40  $\mu$ M. Experiments were performed in aqueous MeCN solution (H<sub>2</sub>O : MeCN = 5 : 95, v/v) with citrate (40  $\mu$ M).

The higher differences in the fluorescence of the enantiomeric iminoboronates is reflected in the linear discriminant analysis (LDA) plot as a greater distance between the clusters

corresponding to amine enantiomers (Fig. 6). LDA is a supervised pattern recognition method, which performs two tasks: First, it reduces dimensionality of the multi-dimensional response data sets, and second, it carries out the classification of the multivariate data.<sup>28,33</sup> LDA models the similarity by calculating the maximum distance between the clusters and the minimum distance between the individual datapoints within each cluster. Furthermore, the cross-validation routine utilizes a two-step procedure comprising model development and model testing to establish the degree of correct classification of the datapoints. The LDA has been employed to test sample clustering and classification. LDA demonstrates that this new assay could discriminate all the analytes including the control. The jackknife cross-validation yields a complete correct classification of all 23 samples corresponding to 11 enantiomeric pairs of analytes and the control. The assay successfully resolved the amine derivatives and organized those into three different groups: amines, amino acid esters and amino alcohols. Significantly, these results prove the assay to be fully capable of discriminating chiral composition in amines, amino esters, and amino alcohols.

The qualitative analysis was then followed by semi-quantitative experiments focused on determination of the enantiomeric purity of analytes with varying ratios of *R/S*- enantiomers. To show that this new method could differentiate between aliphatic amines, which were not distinguished by the original approach,<sup>28</sup> we performed a semi-quantitative analysis of enantiomeric mixtures of 2-aminobutane and valine methyl ester samples (Fig. 7). LDA of the mixtures of analyte enantiomers reflects the changes in fluorescence response to the proportion of enantiomers in the samples. The respective linear discriminant analyses was able to completely and correctly classify (100%) of the enantiomeric compositions and display a highly predictable trend in the position of the analytes depending on the *e.e.* values.

The smooth trend and predictable behavior of the data in the semiquantitative analysis indicated that the system had the potential for success in quantitative analyses and for the determination of the enantiomeric excess in samples of unknown enantiomeric composition. Using 13 data points for calibration and 2 data points for validation of samples with unknown enantiomeric composition, linear regression analysis utilizing support vector machine (SVM)<sup>28,33</sup> was performed (Fig. 8). By definition, SVM is a supervised classification method based on separating classes by mapping the response data into an *n*-dimensional space utilizing kernel functions. The individual datapoints are separated in the *n*-dimensional feature space. The SVM regression develops calibration models serving to predict the samples of unknown *e.e.*. Here, the SVM-based linear regression of the trials with varying enantiomeric composition performed a prediction for enantiomeric compositions of 2-aminobutane and valine methyl ester. This array-based assay resulted in an accurate prediction of the sample behavior. The calculated root mean square error for prediction was < 1.9%.



**Figure 8.** Linear regression for determination of e.e. in the samples of 2-aminobutane (**Top**) and valine methyl ester (**Bottom**). Root mean square errors of prediction (RMSEP) of 1.9 % relates to the error with two independent unknown samples. [FPBA] = 40  $\mu$ M, [Diol dye] = 40  $\mu$ M, [analyte] = 40  $\mu$ M. These experiments were performed in aqueous MeCN solution ( $H_2O$  : MeCN = 5 : 95, v/v) with citrate (40  $\mu$ M).

## Conclusions

In summary, molecular self-assembled systems utilizing boronate esters have been successfully applied to sensing of enantiomeric excess in chiral amines, amino esters, and amino alcohols. These self-assembled complexes comprise axially chiral diol-dyes, formylphenylboronic acid, and amine analyte.

The current method is based on the differential destabilization of the iminoboronate dynamic covalent bond by addition of water and citric acid which causes a change in the esterification equilibrium. This differential destabilization of the iminoboronates is reflected by smaller values of the apparent association constants of one of the two diastereomeric iminoboronates and corresponding amine enantiomer. This method allows the differentiation of difficult to separate amine and amine derivative enantiomers.

The structures of the iminoboronate complexes were investigated by single-crystal X-ray diffraction, ESI MS and DFT calculations. The fluorescence titration experiments demonstrated that the fluorescence changes depend on the structure and chirality of the analytes. The fluorescence output data from microarrays yields a cross-reactive output with information density sufficient to determine the enantiomeric composition in the samples. LDA indicated that the response was able to distinguish and differentiate enantiomers of chiral analytes with 100% correct classification.

Lastly, semi-quantitative and quantitative analyses aimed at the determination of the enantiomeric compositions of chiral amine derivatives in aqueous media were carried out. The SVM-based method for quantitative determination of the enantiomeric composition of 2-aminobutane and valine methyl ester demonstrated that the array-based assay correctly differentiates mixtures with different enantiomeric excess and correctly predicts the e.e. for unknown samples with a high accuracy (a root mean square error of prediction, RMSEP < 1.9%). Experiments described herein are among the most accurate determination of e.e. using fluorescent probes.

Taken together, the results obtained indicate that self-assemblies based on boronate esterification exhibit great potential as fluorescent probes for the determination of enantiomeric excess of chiral amines and derivatives in a high-throughput manner.

## Experimental Section

Analyte mixtures for the array experiments were pre-mixed from 8.8 mM acetonitrile solutions of chiral ligand (S)-BINOL or one of its two analogues (S)-VANOL and (S)-VAPOL, with 8 mM solution of 2-formylphenylboronic acid at 1.1:1 equiv. ratio to reach a final concentration of 4 mM. Subsequently, different amounts of analyte solution as well as additives were added to the solution in order to reach a total volume. For the control experiments, an equal amount of blank solution was added. The total amount of water in acetonitrile was adjusted in every sample for each experiment. The final mixtures were then incubated for 90 min in an Eppendorf Thermomixer R at 25°C to allow the complex formation. The incubated mixtures were dispensed using a high-precision 16-channel pipetting system Nanodrop Express, and then diluted 100 times using BNX 1536™ liquid handling system in 384-IQ-EB Imager Quality Evaporation Barrier Aurora Biotechnologies Microplates. A final concentration of 40  $\mu$ M was obtained. 24 repetitions were performed for each analyte.

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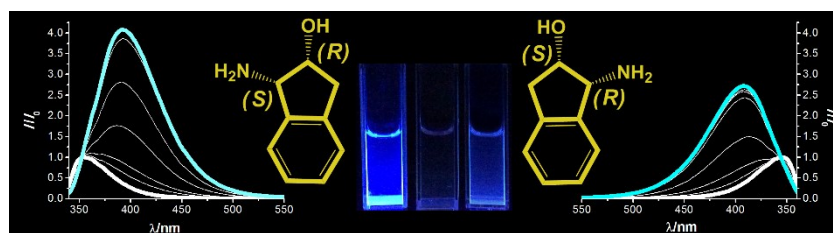
**Keywords:** Supramolecular chemistry • Sensors • Self-assembly • Fluorescence • Chirality



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**Page No. – Page No.**  
Toward Fluorescence-Based High-Throughput Screening for Enantiomeric Excess in Amines and Amino Acid Derivatives